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Troglomorphic features of *Astroblepus pholeter*, a cavefish from Ecuador, and possible introgressive hybridization

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Abstract

Cave organisms are often characterized by reduced pigmentation, eyesight, and enhanced mechanosensory functions. The stygobitic catfish *Astroblepus pholeter* is found within some subterranean drainages in Ecuador. The species was first described in 1962 with specimens that were all highly depigmented and troglomorphic. The next observations in the field occurred until 2011, 2015 and 2018. At such dates, specimens examined progressively displayed more surface-like appearance. Appendages in these individuals were progressively shorter and pigmentation levels are now as high as some surface *Astroblepus*. Based on sampled specimens, it would appear that since 1962, the population has been progressively composed of less troglomorphic individuals. One possibility is that the population has undergone introgressive hybridization in recent years as surface *Astroblepus* are known to enter the caves and cohabitate with the troglomorphic *Astroblepus*. Lastly, we report that Individuals are able to detect and respond to light. Histological analyses show that *A. pholeter*'s eyes have all of the major ocular structures (lens, optic nerve, and all retinal layers).

Keywords

Astroblepus pholeter, cave, catfish, Jumandi cave, troglobite, reduction in pigmentation, introgression

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Introduction

The regression of ancestral features is a frequent event throughout the evolutionary history of organisms when a feature is no longer advantageous. Instances of this occurrence in nature include the loss of teeth in birds (Chen et al. 2000), pelvic and hind limbs in whales (Bejder and Hall 2002), as well as dermal pigmentation and eyes in cavefish (Jeffery 2001; McCauley et al. 2004). Troglobites are an exceptional model in which to study regressive evolution (Jeffery 2001). Many separate cave species have undergone convergent evolution and have developed similar traits such as eye regression, reduction in pigmentation, and enhanced mechanosensory systems, all due to the conditions of living within a cavernicole environment (Jeffery 2001).

Astroblepus pholeter is a stygobiotic catfish from Ecuador. Its type locality is Jumandi Cave. Jumandi is a touristic cave with an outflowings river. Tourists are only able to go a short distance inside the cave, but the cave continues for over 700 meters. The river has turbulent waters, but also contains deep, slow-moving pools. Little is known about this species as only a few related articles have been published. *Astroblepus pholeter* was described as "essentially unpigmented", "pinkish-white in life; lacks bands, spots, or other pigmentation" and is "minute-eyed" when first described by Collette (1962). Haspel et al. (2012) also showed that *A. pholeter* has very few neuromasts, which are mechanosensory structures abundantly found amongst cavefish species. Instead, *A. pholeter* has structures called denticles, which are placoid scales that extend from the surface of their skin. It was suggested (Haspel et al. 2012) that these denticles may have an enhanced troglomorphic function, as these denticles can help to detect the current and position in the cave environment.

Astroblepus pholeter inhabits other caves within the same karstic area (Hoese et al. 2015). A closely related surface morph of *Astroblepus* of an as of yet undetermined species is sometimes found cohabitating inside the caves with the troglomorphic morph (Espinasa et al. 2018). In this study we report on some of the troglomorphic features presented by sampled specimens of *A. pholeter* and their possible variation through time, in an effort to better understand the species and cave adaptation in general.

Methods

Specimens

As is the case of many cave adapted organisms, the type locality of *A. pholeter* at Jumandi Cave, north of the town of Tena, Ecuador, may host a relatively small population, based on visual density of fish compared against classic localities of the Mexican blind tetra, *Astyanax*, and based on the fact that to date, in all collecting efforts authors obtain just a handful of specimens. Collections of cave organisms, such as from this species, should be reduced to a minimum so as not to have a negative impact on fragile populations. For this specific study, no new specimens were col-

lected and those used were described in Collette (1962), Haspel et al. (2012), and Espinasa et al. (2018).

The holotype and two paratypes of *Astroblepus pholeter* (Accession Num 236241; Ecuador, Jumandi cave, 28/Jan/1962), and two surface specimens of *Astroblepus* sp. (MEPN 1649; Ecuador, Napo drainage, River Pucuno, 13/9/2003, and MEPN 1605; Ecuador, Napo province, River Granadilla) kept at the American Museum of Natural History (AMNH) in New York, were examined under a dissection microscope.

In July of 2011, twelve specimens were collected by the research team of one of us (DS). The purpose of that collection did not involve a review of the pigmentation levels of the population, so photographs taken in the field with standardized illumination of each individual are not available. Likewise, specimens in the laboratory were processed in such a way that they are less than adequate to ascertain levels of troglomorphic features for each of the 12 individuals. Nonetheless a general assessment of the population could still be performed. Collecting permit "Permiso de investigación No. 021-IC-FAU/FLO-DPN/MA".

On December 25–27, 2015, six live *A. pholeter* specimens were observed by two of us (LE and JR) while in the field. Luis Alberto Chacha Guayña, a leader of the local community, a guide of the touristic portion of Jumandi cave, and a professional caver captured two of them, photographs were taken in the field, and one was returned unharmed immediately afterwards. The remaining specimen was used for studies in Espinasa et al. (2018) and this same specimen was used in this study in order to reduce the impact of the study on this population. A specimen of *Chaetostoma microps* Günther, 1864, which is within the same order as *A. pholeter*, was also collected from a local surface stream outside of Tena, Ecuador on the 26th of December, 2015 for the purpose of structural comparison to the stygobitic catfish.

On July 2018, five live fish were collected by GH, TT and RT, photographs were taken, and returned unharmed. Two came from Cueva Silvario and three from Cueva Uctu Iji Changa. Both caves are in the same karstic area as Jumandi Cave.

Behavior

The single live specimen of *A. pholeter* captured in 2015 was used for behavioral studies, which were performed within 24 hrs of collection. The specimen was transferred into a two-meter long, 25 cm wide, and 10 cm deep, glass fish tank, with water from the original source. Initial testing was performed on the ability of *A. pholeter* to respond to light through targeted illumination of the head with a StenLight S7 at its highest power-setting. Immediate reactions to the light were recorded. A second behavioral study was then performed where one-half of the tank was covered and kept in darkness while the other half of the tank was fully illuminated with indirect daylight and a 100 watt light bulb 2 m above the tank. The specimen was left in the tank for 12 hours in order to acclimatize prior to data collection. In order to measure the light/darkness preference of the specimen, the individual was then monitored continuously for 3 hours, and the

amount of time spent on the illuminated side of the tank as compared to how much time was spent on the dark side of the tank was recorded. This procedure was then repeated after switching the dark and illuminated sides of the tank and a 30-minute re-acclimatization period. In order to determine if the period of time spent in the illuminated side of the tank was statistically random, a two tailed T-test was performed.

Morphology

In order to determine the structural components of the eyes, the 2015 collected specimens of both *A. pholeter* and *C. microps* were fixed with 90% formol. One eye from each specimen was dissected with the help of a dissection microscope, scalpel, and dissecting needles. Eyes were then embedded in paraffin and cut into 5-µm sections using a microtome. Staining was done with hematoxylin-eosin (Espinasa et al. 2018).

Relative pigmentation levels were assessed visually from live specimens, available photographs, and preserved specimens. As each group's coloration could not be measured under standardized conditions and appearance of coloration is known to vary depending on time of exposure to light in live individuals, photographic techniques, or methods of preservation, no attempt was made to give numerical values for levels of pigmentation, but instead only broad generalizations could be provided.

Length of maxillary barbels and pectoral fins were measured with calipers from preserved specimens or available photographs. As some photographs did not have a scale, data is provided as the ratio of length of barbels or fins divided by the standard length of the specimen.

Results

Pigmentation

Examination of holotype and paratypes stored at the AMNH corroborated the assertion that these specimens were "essentially unpigmented" and "pinkish-white in life", which "lack bands, spots, or other pigmentation" (Collette 1962). No evidence of pigmentation was evident on their body, with the exception of the eyes. Preserved specimens appeared to be marble white in color (Fig. 1–1962). Other surface *Astroblepus* held at the AMNH showed distinct amounts of pigmentation throughout their dorsal surface. This indicates that bleaching caused by preservatives is not the reason for the cave specimen's white coloration, suggesting that while alive, the types were most likely fully depigmented.

The 12 individuals collected in 2011 were variable in color, from almost not pigmented (Fig. 2A) to pigmented (Fig. 2B). As few as two and as much as six were pinkish-white with very little coloration as in figure 2A. This observation was not quantified as it was not the focus of Haspel et al. (2012). Moreover, non-pigmented animals gradually added pigment in response to light and possibly other environmental factors,

Figure 1. Specimens collected in 1962 (holotype, and paratypes of *A. pholeter* from left to right) 2011, 2015 and 2018. Notice that there is a progressive reduction in the expression of troglomorphic features. After 2011 the skin was distinctly more pigmented and the barbels and fins were shorter. Eyes are also embedded under a thinner dermal layer of skin. In 2011 the population was highly variable.

once taken out of the cave. Preserved specimens varied in coloration from marble white to slightly tan (Fig. 1–2011), corroborating that the 2011 specimens showed diversity in their pigmentation levels regardless of photographic conditions of illumination/exposure, with some individuals more pigmented than the 1962 preserved specimens.

While all six specimens observed in 2015 had some degree of depigmentation when compared to average surface fish (Fig. 3), the 2015 observations of live specimens in their native environment gave drastically different results from previous reports. Not a single pinkish-white individual was observed. All 2015 individuals had pigmentation on their skin and a tan coloration (Fig. 1–2015 and 2C) and their dorsal skin (Fig. 2C) was darker than the ventral skin (Fig. 5). This darkness was not uniform throughout the body but instead was localized to certain areas (Fig. 3B; note difference between the cheek area, top of the head above the eye, and above the pectoral fin). A specimen that was submitted to

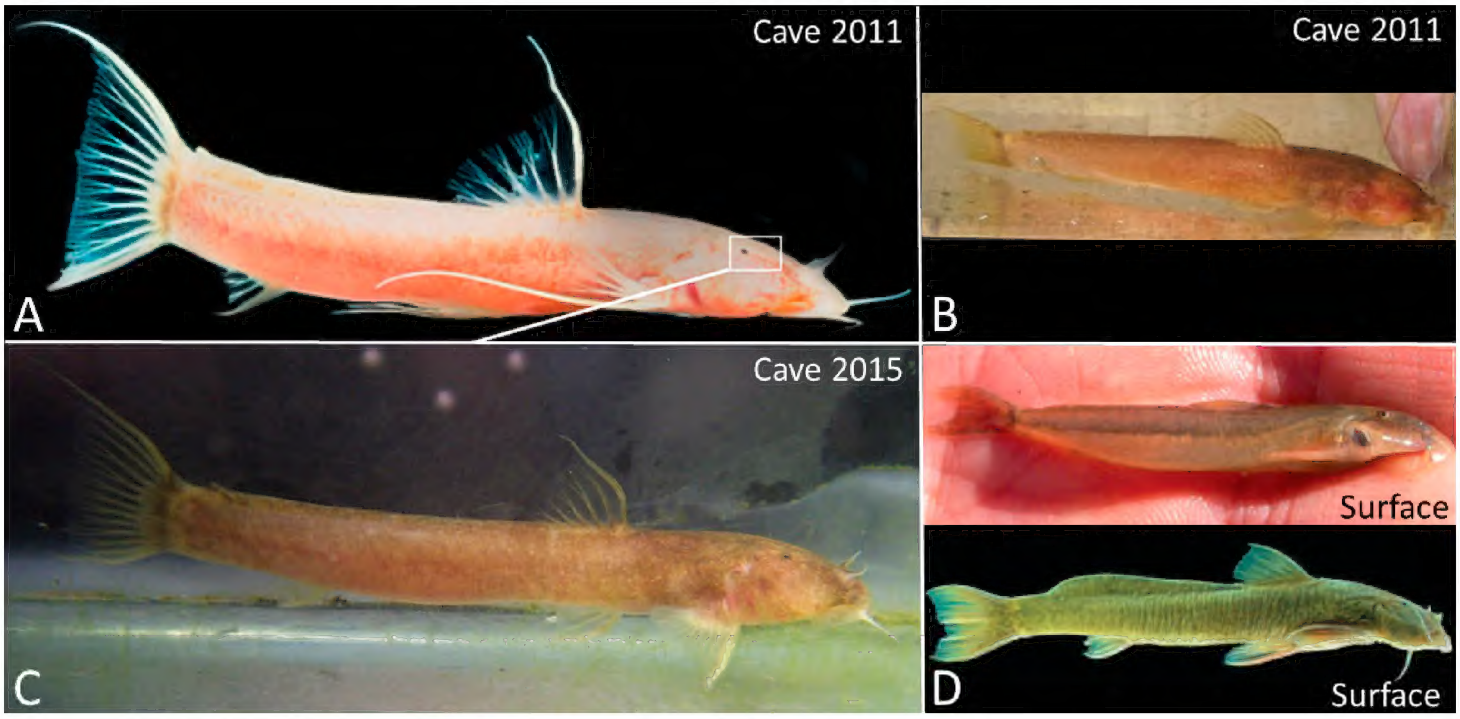

Figure 2. A–C Live specimens of cave *Astroblepus pholeter* **D** Two different surface *Astroblepus* sp. from the Apurimac drainage. Notice contrasting coloration between **A** and **B** both of which were collected in 2011. Pigmentation level variability within the cave population in 2011 spanned from pinkish-white white (**A**) to pigmented (**B**) at levels equivalent to some surface *Astroblepus* (**D**). Specimens collected in 2011 had longer fins (**A**) than those collected in 2015 (**C**). Notice as well that some surface *Astroblepus* (bottom one in **D**) can have small eyes of a size equivalent to cave specimens. **A** Modified from Haspel et al. (2012) **B** Modified from the Soares Lab web-page-photo **C** Specimen collected on 2015 **D** Modified from Schaefer et al. (2011).

sunlight for over 12 hrs also appeared to slightly increase its tan coloration from what was observed in its natural habitat. The single preserved specimen was of a darker coloration than any of the preserved specimens available from 1962 and 2011 (Fig. 1).

The 2018 specimens were the darkest as a group (Fig. 1–2018). One specimen was troglomorphic, with pigmentation levels similar to the 2015 collected specimen. The four remaining specimens had a general appearance consistent with surface fish that may have entered the caves. In a parallel study, Espinasa et al. (2018) showed through 16S sequencing that these four specimens belong to a closely related surface morph.

Regardless of the degrees of depigmentation in the 1962–2018 fish, none of them are albino. Upon examination of their highly reduced eyes, they all appear to be black in color regardless of the year. Histological examination of the eye revealed that the pigmented epithelium in the retina and iris were black and thus must contain functional melanin and are therefore not truly albino (Fig. 4).

Appendage length

A progressive reduction in size of appendages was noticed in the 1962, 2011, 2015 and 2018 specimens. Collette (1962) described the species as having long maxillary barbels which reach to the base of the pectoral fins and with pectoral fins that reach to the anus or slightly beyond (Fig. 5–1962). Available photos and specimens collected in 2011 have

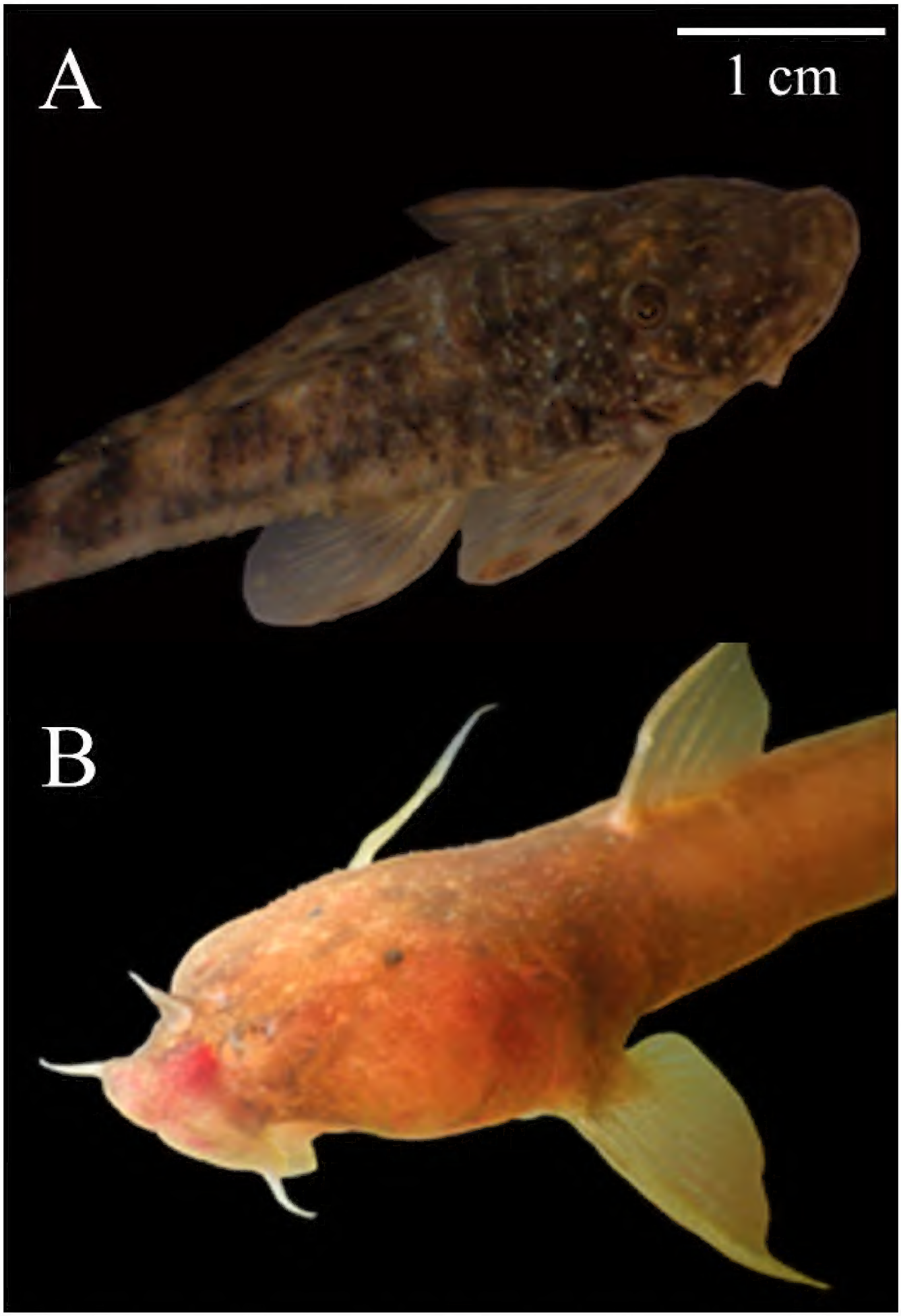

Figure 3. A *Chaetostoma microps* (Surface catfish) **B** *Astroblepus pholeter* (Cave catfish). Both species inhabit the same river drainage.

slightly shorter appendages, but specimens photographed in 2015 and 2018 had distinctly smaller appendages (Fig. 5), where the maxillary barbels reached about half the distance to the pelvic fins and the apex of the pectoral fins reached distinctly short of the anus.

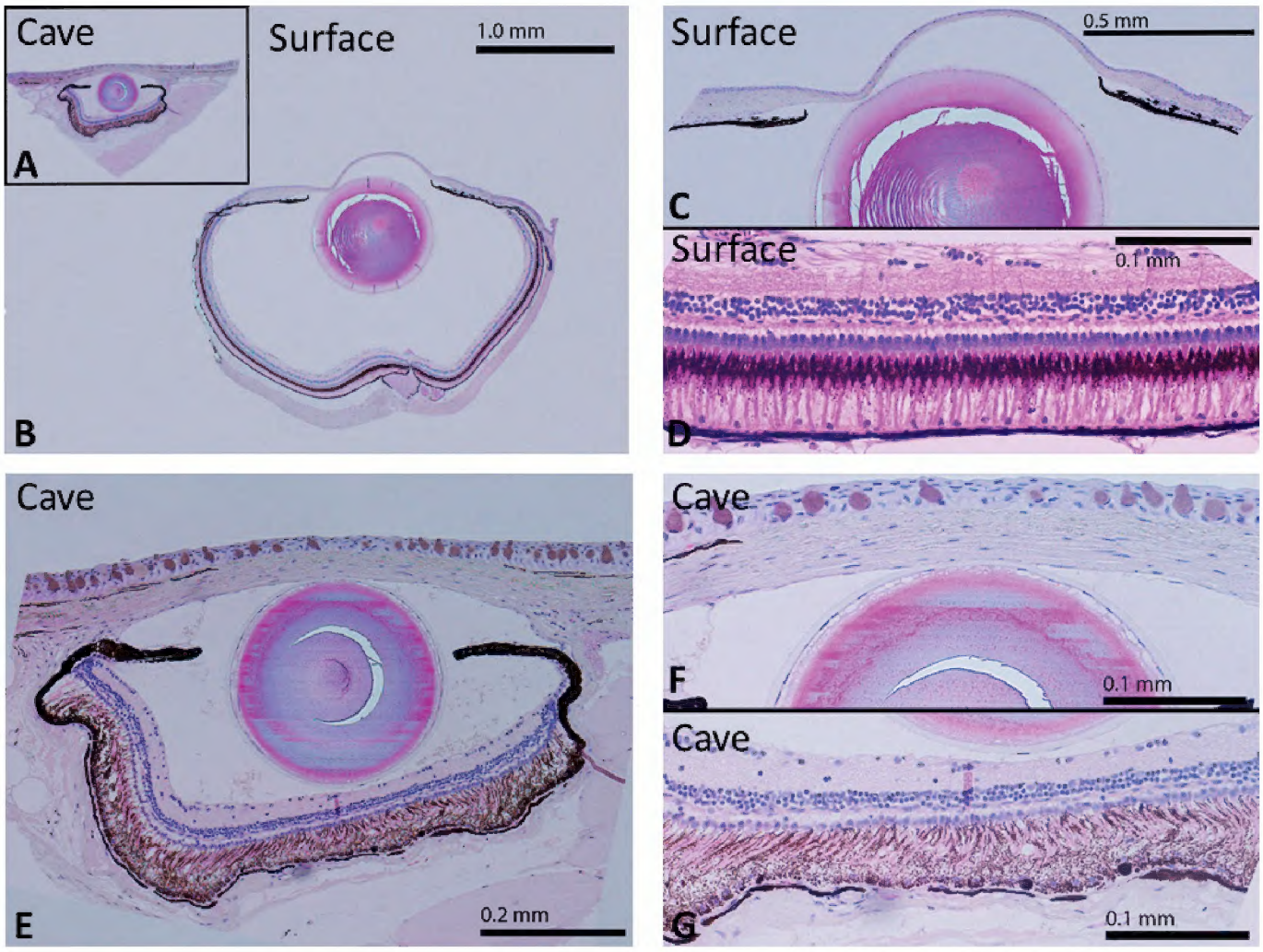

Figure 4. Cross section of the eye from *Astroblepus pholeter* (**A, E–G**) and *Chaetostoma microps* (**B–D**). Notice that the pigmented epithelium (**G**) and iris of *A. pholeter* (**E**) are black, implying that they are not albino. Furthermore, *A. pholeter* eye has retained its lens, optic nerve, and its retina has all the normal layers, as the surface fish eye.

In the 1962 holotype and paratypes the maxillary barbels' length was 22.3–25.9% of the specimens' standard length. In the photographs available of the 2011 collections, the barbels' length was 9.3–13.2%, in the two 2015 specimens with photos available they are 6.3–6.7%, and in the single troglomorphic specimen of 2018 it was 4.4%. The pectoral fins' length on the holotype and paratypes were 38.5–42.7% of the standard body length. In 2011 they were 41.7–42%, in 2015 they were only 16.3–28.2%, and in 2018 20.0%. The four surface morph specimens of 2018 are not included.

Behavior and eye histology

When *A. pholeter* was illuminated in the field directly on the head with a Stenlight headlamp, it reacted immediately with avoidance behavior. When in a 2 m long tank where half of it was in the dark and half of it was illuminated, out of 6 hours it spent 52 minutes (14.4%) of its time in the illuminated side of the tank and 308 minutes (85.6%) in the dark side. This data showed that *A. pholeter* did not randomly position itself throughout the tank ($P < 0.0001$), but rather detected light and preferred the dark side of the tank (nyctophilia).

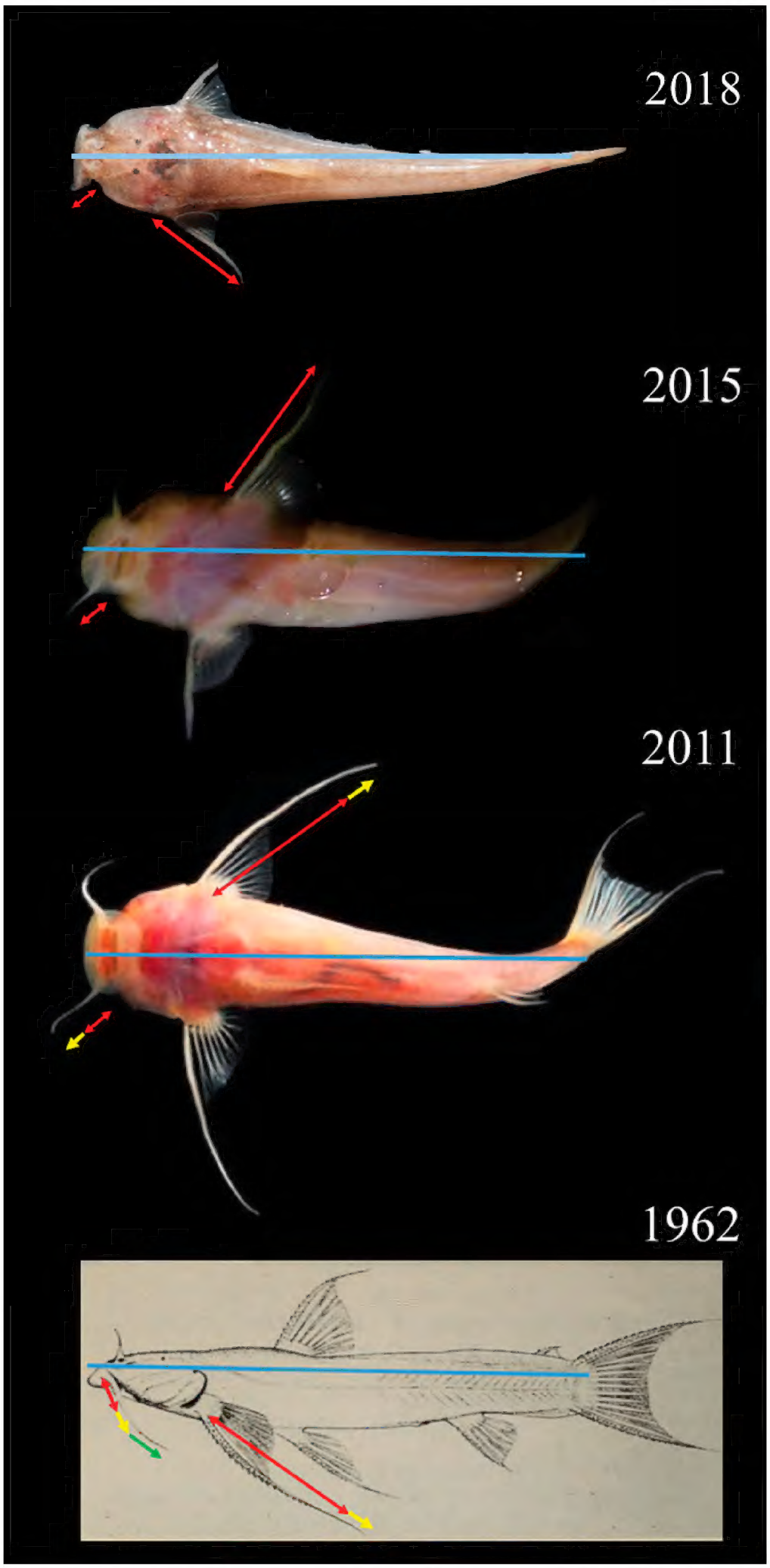

Figure 5. Specimens collected in 1962 (**A** modified from Collette 1962) and 2011 (**B** Modified from Soares and Niemiller 2013) had proportionally longer pectoral fins and maxillary barbels than specimens observed in the field in 2015 (**C**). Images have been scaled to the same body size (blue arrow). Notice that length of appendages in **C–D** (red arrows) are progressively smaller than in **B** and **A** (red plus yellow and green arrows).

Response to light suggested that the specimen was not blind. To further understand if this was true, histological studies of the eye were performed and compared to surface catfish *C. microps*, which is used here as a model of a teleost eye. *Astroblepus pholeter* has much smaller eyes when compared to the surface catfish *C. microps* (Fig. 4A–B). There is an obvious reduction in the size of the vitreous chamber in the cavefish eye. Despite the general reduction of size, the *A. pholeter* eye has retained the lens and optic nerve, and its retina has all of the normal layers as the surface fish eye (Fig. 4D vs 4G). There may be some disorganization within the photoreceptor and pigmented epithelium layers, but the thickness of all retinal layers remains approximately the same as in the surface fish. A clear difference between the surface fish eye and the cavefish eye is that the cavefish eye is embedded under a thick dermal layer instead of a thin cornea (Fig. 4C vs 4F). When the 2015 specimen was compared against the 1962 holotype and two paratypes, the eyes appeared to be embedded under a thinner layer of skin than in the older specimens (Fig. 1).

Despite a severe reduction in eye size, it would appear that all structures necessary for detecting light are present, which may explain the ability of the cavefish to respond to light. The eye being embedded under skin and a reduced vitreous chamber, which positions the lens right next to the retina, probably allows for only blurred and out-of-focus vision. Furthermore, when the eye size in *A. pholeter* was compared against *Astroblepus* sp. kept at the AMNH, it was found that they were comparable in size to some of the smaller-eyed surface *Astroblepus* sp. (Fig. 2D).

Discussion

There are examples of cavefish populations where their troglomorphic features has varied during historical times. Norman (1926) described a cavefish population of the catfish *Rhamdia quelen* in the Cumaca Cave of Trinidad as troglomorphic based on its reduced eye size and pigmentation. Beginning in the 1950s, a number of specimens were collected in the cave showing variability in eye size and pigmentation. Results now suggest that the troglomorphic population has been completely replaced by the epigean morph of the same species in as little as 50 years (Romero et al. 2002). Another example is with the blind tetra, *Astyanax mexicanus*, from Pachón cave in Mexico. Prior to 1986, only albinotic and eyeless fish were observed in this cave. In that year, individual *Astyanax* with variable eye sizes and pigmentation were observed at Pachón. However, in this population the loss of troglomorphic features was only a temporal evolutionary event. Subsequent to this date, the population returned to an albinotic and eyeless condition. The presence of surface fish mitochondrial DNA in Pachón cavefish is also thought to be the result of a fairly recent episode of introgression with surface fish (Langecker et al. 1991). This suggests that contact with surface *Astyanax* fish is a relatively random event that occurs sporadically over time.

Astroblepus pholeter may yet be another population that shows variability in its troglomorphic appearance throughout time. When first described by Collette in 1962, all specimens were highly troglomorphic fish with no skin pigmentation and long

appendages. The 12 individuals collected in 2011 were variable in color, from almost not pigmented to pigmented. At most, half of them had the previous pink/white coloration of the holotype and paratypes. When specimens were observed directly in the field in 2015 from the type locality of Jumandi Cave and in 2018 from neighboring caves, individuals had a drastically different appearance than previously depicted. All combined eleven specimens observed live in the field had distinct dorsal pigmentation and not a single pink/white specimens was observed.

Appendage length, another troglomorphic character, also appears to have reduced. In 1962, the maxillary barbels reached to the base of the pectoral fins and pectoral fins reached to the anus or slightly beyond. Subsequent to 2011, in all specimens collected the maxillary barbels reached to about half the distance to the pelvic fins and the apex of the pectoral fins reached to distinctly short of the anus. Eyes also appeared to be embedded under a thinner layer of skin.

Reduction of troglomorphic features in the population appear to have been progressive through time at the type locality of Jumandi. Based on specimens available, the 1962 specimens were the most troglomorphic, less in 2011, and least in 2015. Although from different caves, the 2018 collection supports this notion because the specimens collected are even less troglomorphic than in 2015. In fact, 80% of the population was composed of the surface morph, based on mitochondrial results by Espinasa et al. (2018). In fact, it appears that the 2011 collection may have coincided when the population structure was undergoing change. The population was variable, with some individuals being highly depigmented, and others as pigmented as some surface *Astroblepus*.

In conclusion, specimens collected of *A. pholeter* at its type locality of Jumandi cave and other caves in the area have displayed progressively a reduced troglomorphic appearance, especially in comparison to the holotype and paratypes when the species was first described. Currently, instead of downright troglomorphic as previously reported, cave populations appear to have increased the number of individuals with high pigmentation levels, reduced length of their appendages, and eyes less embedded under the skin. Since a gradation of phenotypes is observed, and Espinasa et al. (2018) have shown the cohabitation of the surface morph and the troglomorphic morph, it is suggested that perhaps introgressive hybridization with surface specimens, plus perhaps increased migration from surface to cave, have modified the proportion of epigeomorphic specimens collected. Future studies would benefit from a larger sample size (which will always be difficult in these Ecuadorian caves where densities of fish are low), to help understand how much variability there is within the population and how much of this decrease in troglomorphic features is progressive or an oscillatory evolutionary event in which the population will regain its troglomorphic features. Efforts should also be undertaken to collect surface *Astroblepus* that may inhabit the surface stream that emerges from this cave as well as conduct their DNA analyses to corroborate introgression. As of now only surface morph specimens collected inside the cave were available.

Behaviorally, our studies suggest that *A. pholeter* can detect light and has a preference for darkness (nyctophilia). Histological analyses showed that while there may be a reduction in the size of the vitreous chamber and that the eye is embedded under a

dermal layer that may blur images, *A. pholeter* has all of the structures that would allow for vision. This would support that *A. pholeter*'s ability to detect and respond to light may be visually mediated by a functional eye.

Conclusions

We report that specimens collected from *Astroblepus pholeter*, a cave catfish found in caves in Eastern Ecuador, display less troglomorphic features than previously reported. They respond to light and their eyes have a lens, optic nerve, and all retinal layers. Cave specimens currently have pigmentation levels equivalent to some surface *Astroblepus* sp. While available specimens and images are less than adequate to ascertain levels of troglomorphic features, an apparent progressive increase of pigmentation levels as well as a reduction in length of appendages and eye degeneration is evident along 1962, 2011, 2015 and 2018. We suggests that the cave morph of *A. pholeter* may be experiencing introgressive hybridization with a surface *Astroblepus* or increased surface-to-cave migration. The fact that currently surface *Astroblepus* is found living together with troglomorphic *Astroblepus* in some caves gives credence to this hypothesis.

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